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Characterization of an inducible nitrilase from a thermophilic bacillus

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Abstract Nitrilase activity was induced in the thermophilic bacterium *Bacillus pallidus* strain Dac521 by growth on benzonitrile-supplemented minimal medium. The enzyme had a subunit relative molecular mass of 41 kDa but was purified as a complex with a putative GroEL protein (total M_r, 600 kDa). The enzyme catalyzed the hydrolysis of aliphatic, aromatic, and heterocyclic nitriles with widely varying k_{cat}/K_M values, primarily the result of differences in substrate affinity. Of the nitriles tested, 4-cyanopyridine was hydrolyzed at the fastest rate. Substitution of benzonitrile at the meta or para position either had no effect on catalytic rate or enhanced k_{cat}, while ortho-substitution was strongly inhibitory, probably because of steric hindrance. The effect of catalytic inhibitors was consistent with the presence of active site thiol residues although activity was little affected by putative thiol reagents such as iodoacetate, iodoacetamide, and *N*-methylmaleimide. Enzymatic activity was constant between pH 6 and 9 with an optimum at pH 7.6. The optimal temperature for activity was 65°C with rapid activity loss at higher temperatures. The purified nitrilase-GroEL complex had the following half-lives of activity: 8.4 h at 50°C, 2.5 h at 60°C, 13 min at 70°C, and less than 3 min at 80°C.

Key words Benzonitrile · Nitrile · Nitrilase · Thermophilic · Thermostable

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Introduction

Interest in enzymes from thermophilic organisms has been greatly stimulated by the fact that these proteins are resistant to chemical and physical denaturation and to proteolysis (Daniel et al. 1982). The recent expansion of nitrile-dependent biotransformations (Mathew et al. 1988; Nishise et al. 1987; Wyatt and Linton 1988) and enzymatic detoxification of nitrile-based herbicides (Harper 1985; Stalker et al. 1988) and the instability of mesophilic nitrile-metabolizing enzymes (Nagasawa et al. 1993) led us to investigate thermophilic bacteria as an alternative source of these activities (Cramp et al. 1997; Pereira et al. 1998).

The hydration of nitriles in plants and microorganisms is catalyzed by two distinct enzymatic pathways (Nagasawa and Yamada 1989). Typically, aromatic nitriles are catabolized to the corresponding acid in a single-step reaction by a nitrilase. Alternatively, aliphatic nitriles are converted to amides by nitrile hydratases, then hydrolyzed to acids by nonspecific amidases. Exceptions to this exist with *Rhodococcus* sp. (Hjort et al. 1990) and *Bacillus smithii* SC-J05-1 (Takashima et al. 1995) nitrile hydratases and with *Rhodococcus rhodochrous* K22 nitrilase (Kobayashi et al. 1990), all of which can hydrolyze both aliphatic and aromatic nitriles.

Mesophilic nitrilases are relatively unstable, typically undergoing rapid inactivation at temperatures in excess of 50°C (Harper 1985; Kobayashi et al. 1990; Bandyopadhyay et al. 1986). In this study, we present the first reported characterisation of a relatively thermostable nitrilase from a thermophilic microorganism.

Materials and methods

Chemicals

Substrates and inhibitors were obtained from Aldrich (Poole, Dorset, UK); buffer salts, organic solvent and other inorganic chemicals from BDH (England); and other

chemicals from Sigma (Poole, Dorset, UK). Acrylamide/Bis Acrylamide stock solution was obtained from Severn Biotech (Kidderminster, England). Columns and molecular weight standards were from Pharmacia (Uppsala, Sweden).

Bacterial culture condition and nitrilase induction

The bacterial strain used in the study was isolated from hot springs in New Zealand by selective culture on nitriles and has been identified by DNA–DNA hybridization (Cramp et al. 1997) as strain Dac521 of *Bacillus pallidus*. The organism was routinely grown in liquid media consisting of yeast extract (5 g/l), bacto-peptone (5 g/l), K_2HPO_4 (5 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), $FeSO_4 \cdot 7H_2O$ (30 mg/l), and NaCl (1 g/l) pH 7.5 with 5 mM benzonitrile at 50°C. To induce nitrilase activity, a 17% (v/v) inoculum of an active culture was used to inoculate a 500-ml sterile solution consisting of KH_2PO_4 (2 g/l), NaCl (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.2 g/l), $FeSO_4 \cdot 7H_2O$ (30 mg/l) (pH 7.2), and 20 mM benzonitrile as inducer in 2-l flasks. The flasks were shaken at 300 rpm for 20 h at 50°C and the cells were harvested by centrifugation, washed twice with 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM EDTA, and stored at −80°C until required.

Enzyme assay

Nitrilase activity was assayed at 50°C for 15 min in 100-µl assay mixtures that contained 0.1 M potassium phosphate buffer, pH 7.0, 10% methanol, 5 mM EDTA, substrate (100 mM unless stated otherwise), and enzyme (10 µl; ~1 µg). Methanol was added to enhance the solubility of the substrate, the enzyme being stable even in the presence of 10% (v/v) methanol (see following). The ammonia generated was measured by the method of Fawcett and Scott (1960). One unit of activity is defined as the amount of enzyme required to catalyze the formation of 1 µmol/min ammonia at 50°C under standard assay conditions.

Benzamidase and acetamidase activities were assayed essentially as described, but with the omission of methanol from the reaction mixture. Activities are defined as earlier specified.

Protein assay

Protein concentration was determined using the Bio-Rad Bradford protein determination kit with bovine serum albumin (Sigma Fraction V) as protein standard.

Extraction and purification of the enzyme

Acetone fractionation

Frozen cells obtained from 2.5 l of culture medium were thawed and suspended in 100 ml 0.1 M potassium phosphate buffer, pH 7.0, containing 5 mM EDTA. Cells were disrupted by sonicating for 15 min using an MSE 150 W ultrasonic disintegrator at 14 amplitude and centrifuged at 8000 × *g* for 30 min at 4°C. The resulting supernatant (designated as "crude extract") was subjected to acetone precipitation. Cold 100% acetone was added to the enzyme solution to a final concentration of 37.5%. After stirring for 10 min, the precipitate was removed by centrifugation for 20 min at 8000 × *g*. The acetone concentration of the supernatant was increased to 50% by volume and the mixture again stirred for 10 min. The resulting precipitate was collected by centrifugation for 10 min at 8000 × *g* and dissolved in 100 ml of the same buffer. The acetone concentration for the supernatant was increased to 61.5% and the resulting precipitate was collected by centrifugation.

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography of the protein fraction precipitated at 50% acetone saturation was carried out using a 1-ml phenyl-sepharose column (1 × 1 ml; Hi Trap HIC test kit, Pharmacia) preequilibrated with 0.1 M phosphate buffer, pH 7.0, containing 5 mM EDTA. Nitrilase was eluted with a gradient of increasing acetone concentration (0%–20%) at a flow rate of 0.25 ml/min; 2-ml fractions were collected and assayed for enzyme activity. The active fractions were combined and concentrated by centrifugal ultrafiltration (Ultrafree-Cl PBHK; BioMAX-100, Millipore, Tokyo, Japan).

Gel filtration chromatography

Concentrated active fractions eluted from phenyl-sepharose were chromatographed on a Superose 12 HR 10/30 column preequilibrated and eluted at 0.4 ml/min with 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl; 1-ml fractions were collected and assayed for enzyme activity.

Determination of relative molecular mass

Gel filtration chromatography

Concentrated active fractions eluted from phenyl-Sepharose were chromatographed on a Superose 12 HR 10/30 column under nondenaturing (see above) and denaturing conditions. The nondenaturing column was preequilibrated and eluted as previously detailed. The column was calibrated with thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa). Under denaturing conditions, the column was equilibrated and eluted with 0.05 M phosphate buffer, pH 11.5, containing 0.15 M NaCl and 0.1% (w/v) SDS at a flow rate of 0.4 ml/min and calibrated with albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa); 1-ml fractions were collected and assayed for enzyme activity. The void volume was determined with Blue Dextran 2000. The enzyme relative molecular mass values under denaturing and nondenaturing conditions were determined from

semilog plots of the standard protein molecular mass against K_{av} values.

Electrophoresis

Samples were analyzed by SDS-PAGE on 10% acrylamide gels using the Tris/glycine buffer system (Laemmli 1970) and the following M_r standards: phosphorylase b (94kDa), bovine serum albumin (67kDa), chicken ovalbumin (43kDa), bovine carbonic anhydrase (30kDa), soybean trypsin inhibitor (20.1kDa), and bovine α-lactalbumin (14.4kDa). Samples were diluted (4:1) with sample buffer containing 10% SDS and 14.4mM β-mercaptoethanol and heated at 95°C for 5min. For nonreducing conditions, the β-mercaptoethanol was omitted. Proteins were visualized by staining with BM Fast stain (Boehringer Mannheim). The molecular masses of the putative nitrilase enzyme subunits were determined from their relative mobilities, using a plot of the logarithm of the molecular masses of the polypeptide chains of the standard proteins against their relative migration values (R_f).

Amino acid sequencing

Concentrated active fractions from gel filtration chromatography were blotted electrophoretically from SDS-PAGE gels to ProBlott (Applied Biosystems) membranes. The bands were visualized with Coomassie Blue R-250 and sequenced by Dr. Joe Gray of the Molecular Biology Unit, University of Newcastle upon Tyne, using a Beckman LF 3000 microsequencer.

Affinity chromatography

Immunoaffinity chromatography was carried out using a MAb Trap G 11 column (Sepharose-Protein G; Pharmacia). The affinity column was prepared by covalently cross-linking anti-GroEL (Sigma) to sepharose-protein G. The antibodies were passed through the column in 0.2M sodium borate, pH 9, and incubated at room temperature for 1h. The column was then washed and equilibrated with the same buffer containing 20mM dimethylpimelimidate and incubated for 30min at room temperature. The reaction was stopped by washing the column once and equilibrated with 0.2M ethanolamine, pH 8.0, and then incubated for 2h at room temperature. After covalently cross-linking the anti-GroEL with the column matrix, the partially purified nitrilase was mixed 1:1 with 2% *n*-octyl-β-D-glucopyranoside (OGP) (1% final concentration) and passed through the column. The column was washed with 50mM potassium phosphate buffer containing 0.5M NaCl and 1% OGP, and the putative GroEL was eluted with 100mM glycine HCl (pH 2).

Immunodetection

Concentrated active fractions from affinity chromatography were blotted electrophoretically from SDS-PAGE gels to ProBlott membranes. The membrane was soaked in 5% (w/v) nonfat dry milk in 25mM Tris-Cl, 0.5M NaCl, pH 7.5 buffer for 45min at room temperature to prevent nonspecific binding. The membrane was then incubated with the primary antibody (anti-GroEL) in the same buffer for 1h at room temperature and thoroughly washed in 25mM Tris-Cl, 0.5M NaCl, 0.1% v/v Triton X-100, pH 7.5 buffer three times, each for 10min. Incubation with the secondary antibody (horseradish peroxidase-conjugated antirabbit IgG: Amersham Life Science, Buckinghamshire, UK) in TN buffer was carried out for 45min at room temperature, after which the membrane was again washed in TNT buffer as before. The bands were then visualized using the Amersham ECL western blotting detection system.

Determination of stability

Nitrilase samples were incubated at a specified temperature in 0.1M potassium phosphate buffer, pH 7.0, containing 5mM EDTA. At specified time intervals, aliquots were removed and nitrilase activity assayed under standard conditions. Activity is expressed as percentage relative to the activity at time zero (100%).

For solvent stability studies, nitrilase samples (in 0.1M potassium phosphate buffer, pH 7.0, containing 5mM EDTA) were supplemented with solvent to known concentrations, and incubated with 1mM benzonitrile for 15min at 50°C. Activity (as ammonia release) was compared with control (unsupplemented) samples.

Results

Extraction and purification of nitrilase

Nitrilase activity was purified to near-homogeneity from benzonitrile-induced cells as described in Methods. Yields and purification data are given in Table 1, and SDS-PAGE analysis of fractions from the complete protocol is shown in Fig. 1. The clarified cell sonicate was fractionated with acetone (Table 2) and nitrilase activity purified by hydrophobic interaction chromatography. Acetonitrilase and benzamidase activities were not detected after hydrophobic interaction chromatography, but low acetamidase activity was present in active benzonitrilase fractions. Subsequent purification of the active fraction by gel permeation chromatography yielded enzyme preparations free from detectable acetamidase activity. The inclusion of other chromatographic steps (e.g., cation and anion exchange matrices, octyl-sepharose, chromatofocusing on Mono-P, gel filtration in the presence of benzonitrile, and immunoaffinity adsorption chromatography) in the protocol failed to improve the purification and resulted in substantially reduced enzyme yields.

Relative molecular mass and subunit structure

The relative molecular masses of the native enzyme and constituent subunits were determined by gel filtration on

Table 1. Purification of *Bacillus pallidus* Dac521 nitrilase

Step	Protein concentration (mg/ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Crude cell extract	4	504	15	0.03	1	100
50% acetone	5.5	276	16	0.058	1.9	107
Phenyl-sepharose chromatography	0.99	15.8	12	0.76	25	80
Gel filtration	0.103	9.1	9.7	1.06	35	64

Table 2. Yield of nitrile-metabolizing activity in acetone fractions

Acetone (% v/v)	Yield (%)[a]			
	Benzonitrilase	Benzamidase	Acetonitrilase	Acetamidase
0	100	100	100	100
37.5	3 ± 0.7	0.33 ± 0.02	1.8 ± 1.1	7 ± 3
50	115 ± 6	2 ± 0.4	16 ± 3	58 ± 18
61.5	1.7 ± 0.2	37 ± 7	78 ± 6	20 ± 6

[a] Yields are expressed relative to the activities in the cell extract: 100% = 12.7 ± 1.7 units benzonitrile-degrading activity, 35.8 ± 4.0 units benzamide-degrading activity, 9.3 ± 2.3 units acetonitrile-degrading activity, and 5607 ± 636 units acetamide-degrading activity, assayed as described in Methods

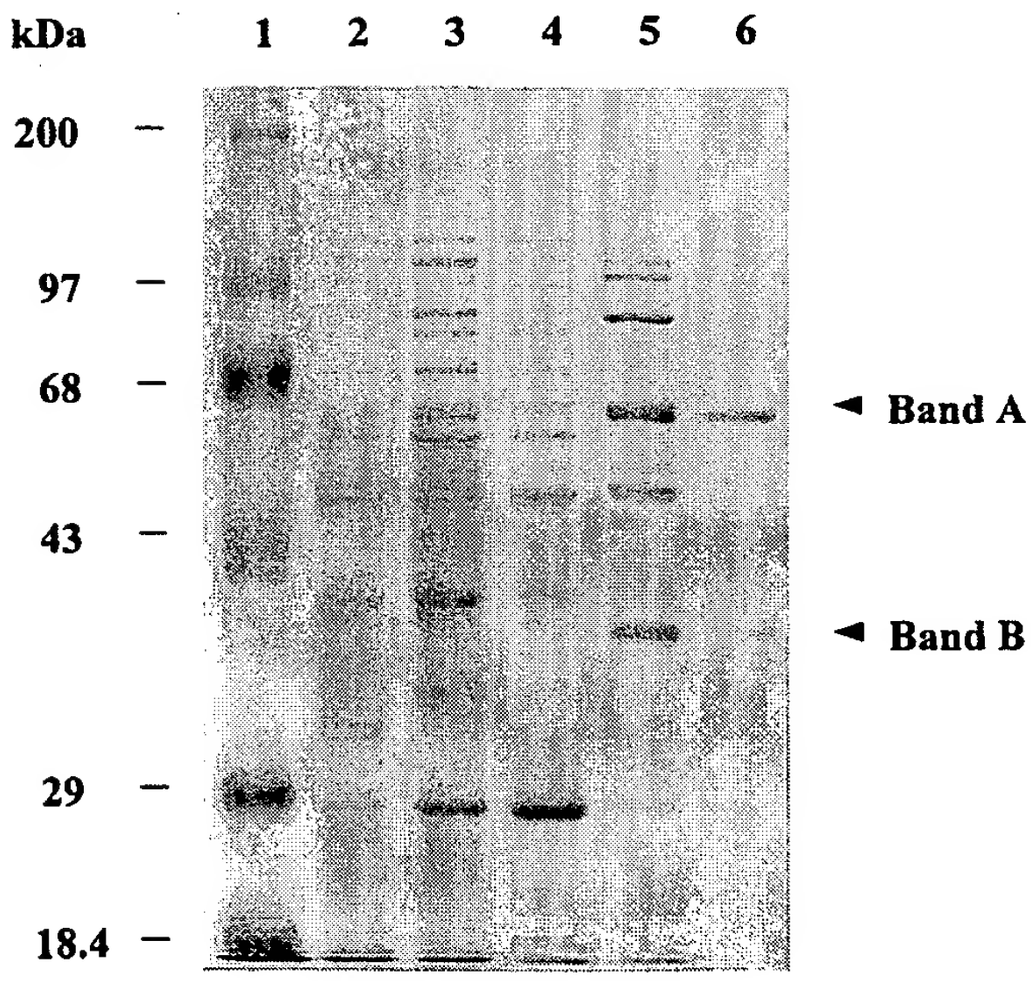

Fig. 1. SDS-PAGE of nitrilase fractions. *Lane 1*, molecular mass standards; *lane 2*, noninduced cell-free extract; *lane 3*, induced cell-free extract; *lane 4*, 50% v/v acetone fraction; *lane 5*, phenyl-sepharose eluate; *lane 6*, superose 12 HR eluate

Superose 12 HR under nondenaturing and denaturing conditions. The elution volume of the active peak from the Superose 12 HR column, under nondenaturing conditions, corresponded to a relative molecular mass of ~600 kDa. Under denaturing conditions, the elution volume of the enzyme corresponded to a relative molecular mass of 44.7 kDa, suggesting that the native enzyme was composed of 14 subunits of similar molecular mass. However, the active eluate from Superose 12 HR under nondenaturing conditions migrated on SDS-PAGE as two major bands with relative molecular masses of 72.4 ± 1.1 and 40.9 ± 1.1 kDa under reduced conditions (Fig. 1) and as also two major bands of 58.5 ± 1.2 and 45.3 ± 1.7 under nonreduced conditions (data not shown).

Amino-terminal sequences

The amino-terminal amino acid sequencing of the two SDS-PAGE bands derived from active gel filtration fractions are shown in Fig. 2a,b. The sequence for band A showed almost complete identity to the sequence of the GroEL protein from *B. subtilis* Marburg (Tozawa et al. 1995) (Fig. 2a). The sequence for band B showed high homology to the sequences of a number of bacterial nitrilases including *Rhodococcus* ATCC 39484 (Stevenson et al. 1992), *R. rhodochrous* strains J1, PA-34, and K22 (Bhalla et al. 1995; Kobayashi et al. 1992a,b), *Klebsiella pneumoniae* subsp. *ozaenae* (Stalker et al. 1988), *Acinetobacter* sp. AK226 (Yamamoto and Komatsu 1991), *Alcaligenes faecalis* strains JM3 and ATCC 8750 (Nagasawa et al. 1990; Yamamoto et al. 1992), and *Comamonas testosteroni* sp. (Levy-Schil et al. 1995) (Fig. 2b). The overall homology of this short protein sequence, and particularly the presence of the VAAVQA consensus region, allows us to conclude with reasonable confidence that this is derived from the nitrilase monomer.

On the basis of the gel permeation chromatography and SDS-PAGE relative molecular mass determinations alone, a native composition of α_{14} for *B. pallidus* DAC521 nitrilase might be assumed. However, the presence of a second pro-

Fig. 2a,b. Amino-terminal amino acid sequences obtained from active nitrilase fractions from gel filtration. **a** Band A compared with *Bacillus subtilis* GroEL protein N-terminal sequence. **b** Band B compared with N-terminal sequences from nitrilases isolated from mesophilic bacteria: *Rhodococcus* ATCC 39484, *R. rhodochrous* J1, PA-34 & K22, *Klebsiella pneumoniae* subsp. *ozaenae*, *Acinetobacter* sp. AK226, *Alcaligenes faecalis* JM3 & ATCC 8750, and *Comamonas testosteroni* sp. Identical residues are indicated as *bold letters*; *X* denotes a residue of unknown identity

a)	
B. pallidus Band A	**A K E I K F S E E A Y R ? A M L C ? G V D X L X D X V ?**
B. subtilis Marburg	**M A K E I K F S E E A R R A M L R G V D A L A D A V**
(b)	
B. pallidus Band B	**S D F S N E K F T V A A V Q A**
Rhodococcus ATCC 39484	**X V E Y T N T F K V A A V Q A Q P V W F D A A K T V D K T**
R. rhodochrous J1	**M V E Y T N T F K V A A V Q A Q P V W F D A A K T V D K T**
R. rhodochrous PA-34	**M V E Y T N T F K V A A V Q A Q P V W F**
R. rhodochrous K22	**M S S N P E L K Y T G K V K V A T V Q A E P V I L D A D A T I D K A**
K. ozaenae	**M D T T F K A A A V Q A E P V W M D A A A T A D K T**
Acinetobacter sp. AK226	**V S Y N S K F L A A T V Q A E P V V L D A**
Alcaligenes faecalis JM3	**M Q T R K I V R A A A V Q A A S P N Y D L A T G V D K T**
Alcaligenes faecalis ATCC 8750	**M Q T R K I V R A A A V Q A A S P N Y D L A T G V D K T**
Comamonas testosteroni sp.	**M K N Y P T V K V A A V Q A A P V F M N L E A T V D K T**

tein component in SDS-PAGE analyses of active gel permeation fractions may influence this conclusion (see following).

Immunoaffinity chromatography

Immunodetection of the active fractions, eluted from the immunoaffinity column, showed that the GroEL-like protein was eluted with the nitrilase (the eluate showed nitrilase activity and the putative GroEL band on DS-PAGE gels: data not shown). The coelution of GroEL-like protein with nitrilase suggests that the putative chaperone protein is bound to the nitrilase and therefore that the nitrilase relative molecular mass estimated from gel permeation chromatography is substantially overestimated. Because the primary function of chaperonins is to interact with proteins and peptides (Ellis 1987), their purification is often a challenging process. It has been claimed that the separation of GroEL protein from other proteins and peptides can be accomplished by including 20% methanol in gel permeation chromatography buffers (Joachimiak et al. 1997). No change in the elution behavior of *B. pallidus* nitrilase was observed under such conditions (data not shown).

Substrate specificity and kinetic behavior

Various nitriles were tested as substrates at a concentration of 100 mM in the presence of 100 mM urea, added to the reaction mixture to inhibit residual amidase activity (Thalenfeld and Grossowicz 1976). The data (Table 3) indicate that *Bacillus pallidus* nitrilase has a very broad substrate specificity, hydrolyzing aliphatic, aromatic, and heterocyclic nitriles. The highest apparent rate was obtained with 4-cyanopyridine. The comparative reactivity for series of substituted benzonitriles (Table 3) suggests that electron-withdrawing ring substituents favor hydrolysis, presumably by enhancing the electropositivity of the nitrile carbon. This general mechanism, supported by the reduced activities on substrates substituted with electron-donating groups, is consistent with a reaction mechanism involving direct nucleophilic attack on the nitrile carbon. A similar trend was observed with electron-withdrawing substituents on aliphatic substrates (e.g., acetonitrile versus chloroacetonitrile; butyronitrile versus 4-chlorobutyronitrile).

With the exception of 2-fluorobenzonitrile, ortho-substituted aromatic substrates, and multisubstituted aromatic halonitriles such as 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil), were hydrolyzed either very slowly or showed no detectable hydrolysis. As the degree of hydrolysis was essentially independent of the chemical nature of the ortho-substituent, this effect may be attributed to steric hindrance as reported for mesophilic nitrilases (Yamamoto and Komatsu 1991; Harper 1977b; Kobayashi et al. 1989).

Bacillus pallidus Dac521 nitrilase preparations gave no detectable activity, determined by release of ammonia, with any amide substrate tested including acetamide, acrylamide, benzamide, 2-cyanoacetamide, fumaramide, nicotinamide, isonicotinamide, propionamide, and *p*-toluamide. In addition, GC analysis of benzonitrile hydrolysis product showed benzoic acid as the sole product. On the basis of these data, we conclude that the enzyme is a broad specificity member of the aromatic-specific nitrilase class, EC 3.5.5.1.

The kinetic parameters of a selection of the most rapidly degraded substrates were determined (Table 4). k_{cat}/K_M values varied widely with different substrates, primarily because of differences in K_M. Substitution of benzonitrile with the chloro group at the meta position decreased both K_M and k_{cat} by approximately an order of magnitude, while para-substitution did not affect either constant to a

Table 3. Substrate specificity[a] of *B. pallidus* Dac521 nitrilase

Substrate	Activity (%)[b] ± SE
ALIPHATIC	
Saturated	
Acetonitrile	2.9 ± 0.3
Chloroacetonitrile	10 ± 0.3
Trichloroacetonitrile	0
Propionitrile	0
Butyronitrile	19.4 ± 0.7
Isobutyronitrile	0
4-Chlorobutyronitrile	109.6 ± 6.6
Valeronitrile	43.3 ± 4
Isovaleronitrile	0
Saturated dinitrile	
Malononitrile	0
Succinonitrile	0
Glutaronitrile	10.9 ± 0.4
Adiponitrile	14.3 ± 2.8
Iminodiacetonitrile	0
3,3-Iminodipropionitrile	0
Unsaturated	
Acrylonitrile	8.9 ± 0.7
Methacrylonitrile	2.4 ± 0.6
Allylcyanide	19.9 ± 1.9
Crotononitrile	80.3 ± 9.8
cis-2-Pentenenitrile	0
trans-3-Pentenenitrile	22.8 ± 2.7
3-Aminopropionitrile fumarate	0
Unsaturated dinitrile	
Fumaronitrile	16.1 ± 2.1
Mucononitrile	2.3 ± 0.4
HETEROCYCLIC	
2-Cyanopyridine	91.6 ± 1.2
3-Cyanopyridine	134.5 ± 1.5
4-Cyanopyridine	232 ± 5
1-Cyclopentene acetonitrile	0
1-Cyanoacetylpiperidine	0
AROMATIC	
Benzonitrile	100
2-Aminobenzonitrile	0
3-Aminobenzonitrile	23.7 ± 1
4-Aminobenzonitrile	3.1 ± 0.7
2-Chlorobenzonitrile	3.2 ± 1.1
3-Chlorobenzonitrile	50.1 ± 4
4-Chlorobenzonitrile	191.5 ± 0.5
2-Hydroxybenzonitrile	0
3-Hydroxybenzonitrile	57.3 ± 3
4-Hydroxybenzonitrile	29.1 ± 1.3
2-Fluorobenzonitrile	27.4 ± 0.6
3-Fluorobenzonitrile	157.2 ± 8.5
4-Fluorobenzonitrile	156.2 ± 6.7
2-Nitrobenzonitrile	2.1 ± 1.1
3-Nitrobenzonitrile	21.2 ± 1.7
4-Nitrobenzonitrile	22.9 ± 2.5
o-Toluonitrile	43.7 ± 2.1
m-Toluonitrile	30.2 ± 0.3
p-Toluonitrile	77 ± 2.4
3-Chloro-4-fluorobenzonitrile	95.2 ± 1.2
3,4-Dibromo-4-hydroxybenzonitrile	0
Phenylacetonitrile	0.7 ± 0.1
4-Aminobenzylcyanide	0
Mandelonitrile	0
2-(Methoxyphenyl)acetonitrile	6.4 ± 1.2
3-(Benzylamino)propionitrile	0
Cinnamonitrile	6.1 ± 0.8
Benzylidenemalononitrile	0

[a] Activity data are derived from total ammonia release during a 15-min period at 50°C and pH 7.0 with a substrate concentration of 100 mM in the presence of 100 mM urea

[b] Activity relative to conversion of benzonitrile under stated conditions

Table 4. Kinetic parameters for selected nitrilase substrates[a]

Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1}mM^{-1}$)
Benzonitrile	0.92	14444	16049
3-Chlorobenzonitrile	0.086	1371	15942
4-Chlorobenzonitrile	1.30	7973	6133
2-Cyanopyridine	0.30	1984	6570
3-Cyanopyridine	2.7	4077	1521
4-Cyanopyridine	22.5	43889	1951
4-Chlorobutyronitrile	41.0	11111	271
Crotononitrile	124.0	7944	64

[a] Substrates were assayed at various concentrations up to 140 mM, and K_M and V_{max} values were calculated from direct linear plots

large extent. The coordinate change in kinetic parameters with the substitutions of aromatic and heterocyclic rings suggests that the electronic configuration of the ring structures affects binding and catalytic efficiency in a differential manner. While the presence of a bulky residue proximal to the cyano group clearly has adverse effects on substrate binding, and the positioning of electron-withdrawing substituents to favor nitrile-carbon electropositivity consistently enhances catalytic rate, the significance of the positioning of the heterocyclic nitrogen with respect to the nitrile moiety in cyanopyridine derivatives is less clear.

Inhibition of nitrilase activity

The effect of various reagents on nitrilase activity is shown in Table 5. Silver and mercuric ions were found to be very powerful inhibitors. Other divalent metal ions had little or no significant effect on enzymatic activity unlike, for example, *Nocardia* sp. NCIB 11216 nitrilase (Harper 1985), which was inhibited by Fe^{2+} and Mn^{2+}. The effects of thiol reagents were reasonably consistent; 4-chloromercuribenzoic acid, *p*-hydroxymercuribenzoic acid, and phenylmercuric acetate and the thiol exchange reagent DTNB were powerful inhibitors, strongly suggesting that one or more thiol residues were involved at the active site. This conclusion is supported by the sensitivity of the enzyme to Ag^{+} and Hg^{+} ions and, to a lesser extent, by the partial inactivation by iodoacetate and iodoacetamide and activation by the reductants cystine and sodium hydrosulfide.

Metal ion chelators such as EDTA, 8-hydroxyquinoline, 1,10-phenanthroline, and sodium azide had little or no significant effect on nitrilase activity, nor would any effect be expected. None of the reported studies of mesophilic bacterial nitrilases implicates metal ions in either the catalytic mechanism or in conformational stabilization. Neither phenylhydrazine, a carbonyl reagent that inhibited the enzyme from *R. rhodochrous* J1 (Kobayashi et al. 1989), nor urea, a potent inhibitor of amidase activity, affected *B. pallidus* nitrilase activity.

Various aromatic compounds were tested at 1 mM concentrations as substrate analog inhibitors. Only benzaldehyde produced strong inhibition (95%) and was selected for more detailed study. Benzaldehyde was found to act as a

Table 5. Effect of various compounds on the activity of nitrilase

Group	Addition[a]	% Relative activity ± SE
Control	None	100
Metal ions	$AgNO_3$	4.0 ± 1.3
	$CaCl_2$	90.3 ± 2.3
	$CuCl_2$	90.0 ± 2.9
	$CrCl_3$	92.3 ± 2.2
	$FeSO_4$	84.0 ± 3.0
	$HgCl_2$	5.5 ± 1.8
Thiol active agents	4-Chloromercuribenzoic acid	0
	DTNB	55.6 ± 2.5
	p-Hydroxymercuribenzoic acid	3.0 ± 1.6
	Iodoacetamide	86.7 ± 2.3
	Iodoacetic acid	75.3 ± 0.7
	Phenylmercuric acetate	0
Chelating agents	8-Hydroxyquinoline	63.8 ± 1.3
	4,7-Phenanthroline	65.7 ± 2.2
Reducing agents	*L*-Cystine	115.7 ± 4.2
	DTT	82.0 ± 4.4
	Glutathione (reduced)	76.7 ± 1.5
	Sodium hydrosulfide	119.0 ± 3.5
	Phenylhydrazine	86.0 ± 1.5

The enzyme was dialyzed exhaustively against 100mM potassium phosphate pH 7.0, incubated with the specified compounds (final concentration 1 mM) and the assay initiated by addition of benzonitrile (1mM). After a further 15-min incubation under standard reaction conditions, the release of ammonia was determined as indicated in Methods. Data are mean values of triplicate experiments

[a]The following compounds gave no significant change in nitrilase activity (at ±3 SE) compared with control experiments: $AlCl_3$, $CoCl_2$, $FeCl_3$, $MgCl_2$, $ZnCl_2$, $MnCl_2$, NaCl, NaF, $NiCl_2$, $PbCl_2$, EDTA, *N*-ethylmaleimide, 1,10-phenanthroline, NaN_3, PMSF, ascorbic acid, 2-mercaptoethanol

competitive inhibitor with a K_i value of 12.4μM, as determined from Dixon plots. Closely related compounds (aniline, benzamidine, benzoic acid, benzyl alcohol, benzamide, and benzamine) showed little (<15%) or no inhibitory effect on nitrilase activity.

Effect of pH on nitrilase activity

The effect of pH on the nitrilase activity was measured under standard assay conditions in 0.1M of citric acid:Na_3 citrate, KH_2PO_4:K_2HPO_4, boric acid:Na_3 borate, and Na_2CO_3 buffers over a pH range of 3 to 11 (Fig. 3a). The enzyme showed a broad peak of activity between pH 6 and 9 with an optimum in the region of 6.5 to 8.0. Rapid inactivation at low pH might potentially be linked with protonation of active site thiol residues.

Effect of temperature on nitrilase activity and stability

The activity of *B. pallidus* Dac521 nitrilase was determined at temperatures over a range of 25°–80°C. The optimal temperature under the reaction conditions used was approximately 65°C; above 70°C, enzyme activity was rapidly lost (Fig. 3b). Replotted in the form of an Arrhenius curve (not shown), the linear portion of the temperature–activity profile between 25° and 60°C gave an activation energy of 36kJ/mol.

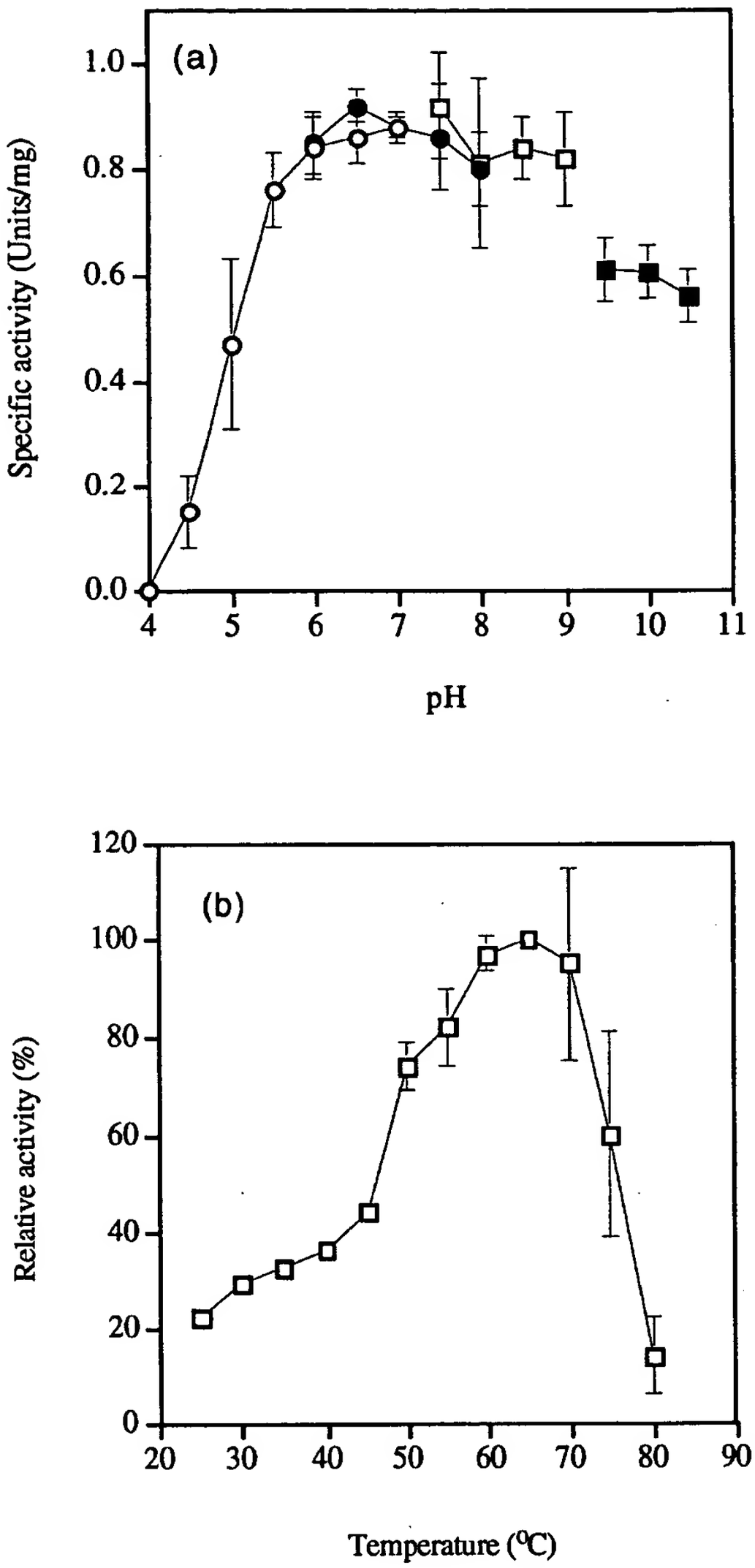

Fig. 3. **a** Effect of pH on the activity of *B. pallidus* Dac521 nitrilase. Reactions were carried out for 15min at 50°C in the following 0.1M buffer solutions: citrate (*open circles*), phosphate (*closed circles*), borate (*open squares*), and bicarbonate buffer (*closed squares*). **b** Effect of temperature on the activity of *B. pallidus* Dac521 nitrilase. Reactions were carried out for 15min at different temperatures. Activities are expressed as percentages relative to the maximum activity at 65°C (100%) under experimental conditions used

The stability of the enzyme was examined at various temperatures (Fig. 4). Under the specified incubation con-

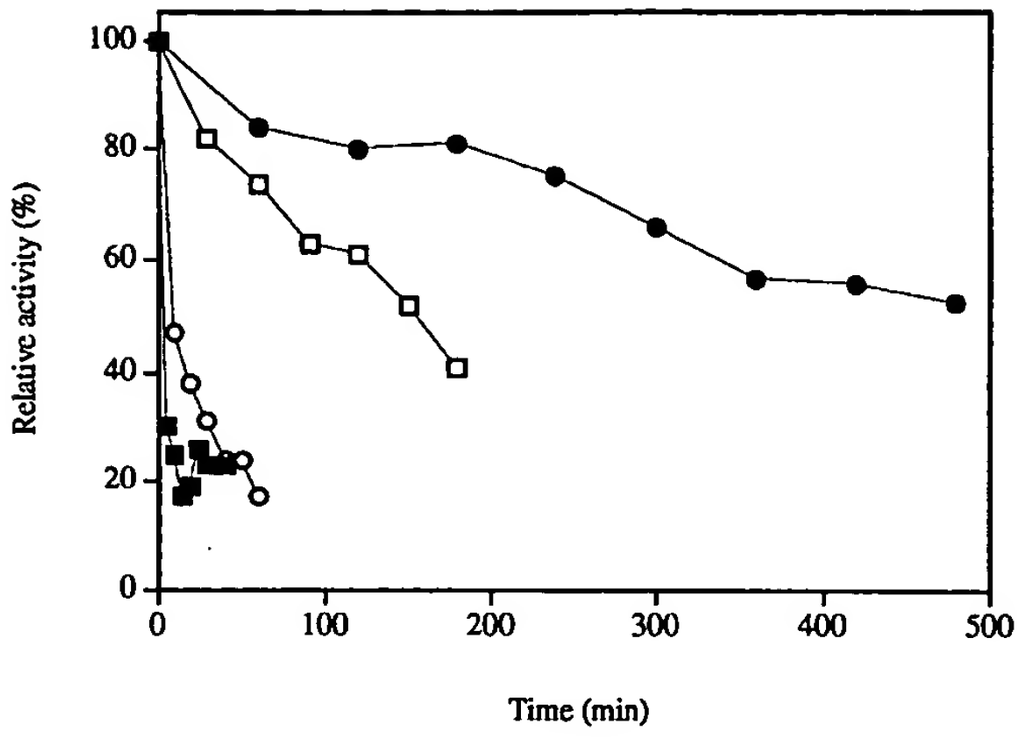

Fig. 4. Effect of temperature on the stability of *B. pallidus* Dac521 nitrilase. Incubations were performed at the following temperatures: 0–8 h 50°C (*closed circles*), 60°C (*open squares*), 70°C (*open circles*), and 80°C (*closed squares*). At specific time points during incubation in 0.1 M potassium phosphate buffer, pH 7.0, containing 5 mM EDTA, aliquots of each enzyme solution were taken and nitrilase activity assayed under standard assay conditions. Activity is expressed as percentage relative to the activity at zero time (100%)

ditions, the nitrilase exhibited the following half-lives of irreversible inactivation: 8.4 h at 50°C, 2.5 h at 60°C, 13 min at 70°C, and <3 min at 80°C.

Effect of organic solvent on nitrilase activity

The effect of different concentrations of organic solutes and solvents on the activity of nitrilase was determined under standard assay conditions. At concentrations between 20 and 30% v/v, ethylene glycol was found to induce a small but significant activation. Hydrophilic organic solvents including acetone, ethanol, and methanol reduced activity over the reaction period (15 min) in a concentration-dependent manner. Little or no activity was detectable in these solvents at concentrations of 50% v/v after incubation for 15 min at 50°C.

Discussion

This study represents the first reported isolation and functional characterisation of a nitrilase from a thermophilic microorganism. The addition of benzonitrile to minimal culture media was found to induce both aromatic-specific nitrilase (benzonitrilase) and benzamidase activities, the two activities being effectively separated by acetone fractionation. Even at relatively high concentrations, acetone had no apparent deleterious effect on the enzyme activity, in accordance with the enhanced solvent stabilities of thermophilic enzymes (Owusu and Cowan 1989). The nitrilase was purified 35 fold from cell-free extracts to near-homogeneity with a final yield of 64%, indicating that enzyme protein was less than 3% of the total soluble protein in benzonitrile-induced cells.

Bacillus pallidus nitrilase was found to have a relative molecular mass of approximately 600 kDa as determined by gel permeation chromatography. With a subunit size of approximately 41 kDa from SDS-PAGE, an α_{14} native structure is proposed, which would be consistent with the quaternary compositions of mesophilic microbial nitrilases. For example, the nitrilases from *Nocardia* sp. strains NCIB 11215 and NCIB 11216 had a native relative molecular mass of 560 kDa with twelve 46-kDa subunits (Harper 1985, 1977b), whereas *Rhodococcus rhodochrous* K22 nitrilase was a 650-kDa enzyme composed of fifteen to sixteen 41-kDa subunits (Kobayashi et al. 1990). However, in light of the evidence that the second protein (identified as a putative GroEL by N-terminal sequencing) in active fractions from gel permeation chromatography was in direct interaction with the nitrilase, the α_{14} composition must be reconsidered. Immunoaffinity purification using immobilized anti-GroEL antibodies resulted in the coelution of the putative GroEL and the nitrilase activity, suggesting an intimate association of the proteins rather than merely coincident elution. The native molecular mass of the nitrilase cannot therefore be readily assigned. Nevertheless, the apparent association of the two proteins raises the interesting speculation that *B. pallidus* GroEL possesses a specific stabilizing function in vivo.

Bacillus pallidus Dac521 nitrilase has been shown to have a very broad substrate specificity, hydrolyzing a wide range of substituted aromatic, heterocyclic, and aliphatic nitriles (see Table 3). This specificity is consistent with the observation that microbial nitrilases typically demonstrate a much wider specificity than nitrile hydratases. The *B. pallidus* enzyme showed a particularly high hydrolytic rate for 4-cyanopyridine, a specificity previously observed for the mesophilic nitrilases isolated from *Fusarium solani* (Harper 1977a) and *Nocardia* sp. NCIB 11215 (Harper 1985).

Benzaldehyde was found to be a powerful competitive inhibitor of *B. pallidus* Dac521 nitrilase (K_i = 12.4 μM). As previously suggested (Stevenson et al. 1992), aldehydes readily react with active site cysteines to form thiohemiacetals, a covalent product that may mimic the second tetrahedral intermediate in the proposed mechanism for nitrilase catalysis (Harper 1977b). However, we note that the kinetics of benzaldehyde inhibition of *B. pallidus* nitrilase were typical of reversible competitive inhibition.

As expected for a thermophile-derived enzyme, both the apparent temperature optimum (65°C) and the thermostability in buffered aqueous solution (88% and 50% activity retained at 50°C after incubation for 1 and 8 h, respectively) were significantly higher than values reported for mesophilic nitrilases. For example, the highest optimal temperature reported for a mesophilic microbial nitrilase was 50°C; above 65°C, the enzyme was rapidly inactivated (Yamamoto and Komatsu 1991). The *Rhodococcus rhodochrous* J1 nitrilase retained only 6.9% of initial activity when incubated at 50°C for 1 h (Kobayashi et al. 1989).

We note however that the thermostability of the "purified" nitrilase will be influenced by the presence of the GroEL-like protein. As the evidence suggests an intimate association between the proteins, we must assume that the thermostability of the homogeneous nitrilase might be significantly reduced. The possible in vivo role of the GroEL-like protein as a functional stabilization agent remains a matter of speculation.

The presence of a coeluting protein in the purified nitrilase preparation might be attributed to coincident chromatographic properties or to some more specific relationship. GroEL protein is one of the best characterized bacterial heat-shock proteins (Ellis 1987). The possible function of the *B. pallidus* GroEL-like protein as a molecular chaperonin and the difficulties in separating this protein from the active nitrilase suggest that this protein may bind with high affinity to the nitrilase. In related organisms, GroEL protein has been found to be required for proper folding and prevention of denaturation of cellular proteins at high temperatures (*Bacillus subtilis*, Chang et al. 1994; alkaliphilic *Bacillus* sp. strain C-125, Xu et al. 1996). The intriguing possibility that GroEL protein binds to *B. pallidus* nitrilase to form GroEL–nitrilase complexes, which could increase the stability of nitrilase at high temperature, requires further investigation.

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References

Bandyopadhyay AK, Nagasawa T, Asano Y, Fujishiro K, Tany Y, Yamada H (1986) Purification and characterisation of benzonitrilase from *Arthrobacter* sp. strain J-1. Appl Environ Microbiol 51:302–306

Bhalla TC, Aoshima M, Misawa S, Muramatsu R, Furuhashi K (1995) The molecular cloning and sequencing of the nitrilase gene of *Rhodococcus rhodochrous* PA-34. Acta Biotechnol 15:297–306

Chang B-Y, Chen K-Y, Wen Y-D, Liao C-T (1994) The response of a *Bacillus subtilis* temperature-sensitive *sigA* mutant to heat stress. J Bacteriol 176:3102–3110

Cramp R, Gilmour M, Cowan DA (1997) Novel thermophilic bacteria producing nitrile-degrading enzymes. Microbiology 143:2313–2320

Daniel RM, Cowan DA, Morgan HW, Curran MP (1982) A correlation between protein thermostability and resistance to proteolysis. Biochem J 207:641–644

Ellis L (1987) Proteins as molecular chaperones. Nature (Lond) 328:378–379

Fawcett JK, Scott JE (1960) A rapid and precise method for the determination of urea. J Clin Pathol 13:156–159

Harper DB (1977a) Fungal degradation of aromatic nitriles: enzymology of C-N cleavage by *Fusarium solani*. Biochem J 167:685–692

Harper DB (1977b) Microbial metabolism of aromatic nitriles: enzymology of C-N cleavage by *Nocardia* sp. NCIB 11216. Biochem J 165:309–319

Harper DB (1985) Characterisation of a nitrilase from *Nocardia* sp. NCIB 11215, using *p*-hydroxybenzonitrile as sole carbon source. Int J Biochem 17:677–683

Hjort CM, Godtferedson SE, Emborg C (1990) Isolation and characterisation of a nitrile hydratase from a *Rhodococcus* sp. J Chem Technol Biotechnol 48:217–226

Joachimiak A, Quaite-Randall E, Tollasksen S, Mai X, Adams MWW, Josephs R, Giometti C (1997) Purification of chaperonins from thermophilic bacteria and archaea. J Chromatogr A 773:131–138

Kobayashi M, Nagasawa T, Yamada H (1989) Nitrilase of *Rhodococcus rhodochrous* J1: purification and characterisation. Eur J Biochem 182:349–356

Kobayashi M, Yanaka N, Nagasawa T, Yamada H (1990) Purification and characterisation of a novel nitrilase of *Rhodococcus rhodochrous* K22 that acts on aliphatic nitriles. J Bacteriol 172:4807–4815

Kobayashi M, Komeda H, Yanaka N, Nagasawa T, Yamada H (1992a) Nitrilase from *Rhodococcus rhodochrous* J1: sequencing and overexpression of the gene and identification of an essential cysteine residue. J Biol Chem 267:20746–20751

Kobayashi M, Yanaka N, Nagasawa T, Yamada H (1992b) Primary structure of an aliphatic nitrile degrading enzyme, aliphatic nitrilase from *Rhodococcus rhodochrous* K22 and expression of the gene and identification of its active site residue. Biochemistry 31:9000–9007

Laemmli UK (1970) Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature (Lond) 227:680–685

Levy-Schil S, Soubrier F, Crutzlecoq AM, Faucher D, Crouzet J, Peter D (1995) Aliphatic nitrilase from a soil-isolated *Comamonas testosteroni* sp.; gene cloning and overexpression, purification and primary structure. Gene 161:15–20

Mathew CD, Nagasawa T, Kobayashi M, Yamada H (1988) Nitrilase-catalysed production of nicotinic acid from 3-cyanopyridine in *Rhodococcus rhodochrous* J1. Appl Environ Microbiol 54:1030–1032

Nagasawa T, Yamada H (1989) Microbial transformation of nitriles. Trends Biotechnol 7:153–159

Nagasawa T, Mauger J, Yamada H (1990) A novel nitrilase, arylacetonitrilase, of *Alcaligenes faecalis* JM3. Eur J Biochem 194:765–772

Nagasawa T, Shimizu S, Yamada H (1993) The superiority of the third generation catalyst *Rhodococcus rhodochrous* J1 nitrile hydratase for industrial production of acrylamide. Appl Microbiol Biotechnol 40:189–195

Nishise H, Kurihara M, Tani Y (1987) Microbial synthesis of a tranexamic acid intermediate from dinitrile. Agric Biol Chem 51:2613–2616

Owusu RK, Cowan DA (1989) Correlation between microbial protein thermostability and resistance to denaturation in aqueous-organic solvent 2-phase systems. Enzyme Microbial Technol 11:568–574

Pereira RA, Graham D, Rainey FA, Cowan DA (1998) A novel thermostable nitrile hydratase. Extremophiles 2:347–357

Stalker DM, Malyj LD, McBride KE (1988) Purification and properties of a nitrilase specific for the herbicide bromoxynil and corresponding nucleotide sequence analysis of the *bxn* gene. J Biol Chem 263:6310–6314

Stevenson DE, Feng R, Dumas F, Groleau D, Mihoc A, Storer AC (1992) Mechanistic and structural studies on *Rhodococcus* ATCC 39484 nitrilase. Biotechnol Appl Biochem 15:283–302

Takashima Y, Mukumoto F, Mitsuda S (1995) Process for production of amide compounds using microorganism. Patent EP 0666 321 A2

Thalenfeld B, Grossowicz N (1976) Regulatory properties of an inducible aliphatic amidase in a thermophilic bacillus. J Gen Microbiol 94:131–141

Tozawa Y, Yoshikawa H, Kawamura F, Itaya M, Takahashi H (1995) Isolation and characterisation of the *groES* and *groEL* genes of *Bacillus subtilis* Marburg. Biosci Biotechnol Biochem 56:1995–2002

Wyatt JM, Linton EA (1988) The industrial potential of microbial nitrile biochemistry. In: Evered D, Harnett S (eds) Cyanide compounds in biology. Ciba Foundation Symposium, vol 140. Wiley, Chichester, pp 32–48

Xu Y, Kobayashi T, Kudo T (1996) Molecular cloning and nucleotide sequence of the *groEL* gene from the alkaliphilic *Bacillus* sp. strain C-125 and reactivation of thermally inactivated α-glucosidase by recombinant groEL. Biosci Biotechnol Biochem 60:1633–1636

Yamamoto K, Komatsu K (1991) Purification and characterisation of nitrilase responsible for the enantioselective hydrolysis from *Acinetobacter* sp. AK226. Agric Biol Chem 55:1459–1466

Yamamoto K, Fujimatsu I, Komatsu KI (1992) Purification and characterisation of nitrilase from *Alcaligenes faecalis* ATCC 8750 responsible for enantioselective hydrolysis of mandelonitrile. J Ferment Bioeng 73:425–430